

Appl. No.: 09/837,235

Docket No. 289550-122 US2

Amdt. dated May 28, 2004

Reply to Final Office Action dated Mar. 31, 2004

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Amendments to the Specification:

Please replace the paragraph bridging pages 16 and 17, with the following amended paragraph:

FIG. 15. A. Nucleotide and amino acid sequence of the *C. antarctica* Lipase B. Both sequences start ~~where~~ where the 25 amino acid pre-propeptide is cleaved. B. Sequences of oligonucleotides used for cloning, site-directed mutagenesis, and error-prone PCR, as indicated. The pPal-CALB vector is based on the pPICZalphaA vector, whereby the insert is the N-terminally His-tagged reading frame of the CALB gene, as represented in A, that is cloned into the EcoRI and NotI sites in the multiple cloning site of the vector. The vector pYal-CALB is based on the pYES2.1 V5-His-TOPO vector, whereby the insert is the alpha factor--CALB fusion, containing the N-terminal His-tag, EcoRI and NotI restriction sites, amplified from the pPal-CALB vector. Primers for error-prone PCR allow for directional cloning of the PCR product into the EcoRI and NotI sites in the pYal-CALB vector. All of the constructs are generated by single amino acid substitutions.

Please replace the paragraph at page 30, lines 27-32, with the following amended paragraph:

The identified and isolated gene can then be inserted into an appropriate cloning or expression vector. A large number of vector-host systems known in the art may be used. Possible vectors include plasmids or modified viruses, but the vector system must be compatible with the host cell used. Such vectors include bacteriophages such as lambda derivatives, or plasmids such as ~~PBR322~~ pBR322 or pUC plasmid derivatives or the Bluescript vector (Stratagene).

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Please replace the paragraph at page 32, lines 13-25, with the following amended paragraph:

Engineering the overall structure and function of a stabilized polypeptide or polypeptide complex is achieved by controlling the availability of tyrosyl side-chains for the cross-linking reaction, for example, but not limited to, via mutagenesis. Functionality of a polypeptide or polypeptide complex may be compromised or altered by a tyrosine-tyrosine cross-link reaction. In this case, an undesirable hydroxyl group of a tyrosyl side-chain may be removed by mutating such residues to phenylalanine, or ~~masked~~ masked to inhibit its participation in such a reaction. In this way, a tyrosyl residue available for the cross-linking reaction but that may lead to distortion of structure and compromise functionality and/or specificity of the polypeptide or polypeptide complex is removed. Moreover, point mutations to tyrosine may be introduced at positions where the tyrosyl side-chains will react with each other to form a bond that causes the least distortion to structure and function; these positions are identified as described in detail below. Thereby, the overall structure and functionality of the polypeptide or polypeptide complex is maintained.

Please replace the paragraph bridging pages 41 and 42, with the following amended paragraph:

Measurements that can be made to attain information concerning this potential relate to the determinants of the space available for the reactive side-chains and the bond. Such measurements include the distance between the residue pairs' alpha-carbons, which are the carbon atoms that are a part of the "backbone" formed by the peptide bonds between all amino acids of the polypeptide. The selected residue pairs should have an average alpha-carbon distance close to the distance that the alpha-carbons of the cross-linked tyrosyl side-chains would be from each other if point

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mutations were introduced, and the cross-link reaction were directed to that residue pair. The selected residue pairs ~~should be~~ should be so close to the distance of the alpha-carbons of cross-linked tyrosyl side-chains to ensure that the functionality of the polypeptide or polypeptide complex is maintained. The criteria for this selection are described in detail below (Selection Process: Determination of the Alpha Carbon Distance in the Tyrosyl-tyrosyl Bond, The Filters). Since the variability of a residue pair's structural characteristics is also an important criterion in the selection of suitable residue pairs for the cross-link reaction (see below), the required proximity to the optimal distance is calculated for each residue pair, dependent on the variability of its alpha-carbon distances in the sample. The calculation of this requirement is also described in detail below (Selection Process: The Filters).

Please replace the paragraph at page 53, lines 18-23, with the following amended paragraph:

In yet another specific embodiment, the polypeptide, or one, any, both, several or all of the polypeptides of a complex may be expressed with signal peptides, such as, for example, pelB bacterial signal peptide, that directs the protein to the bacterial periplasm (Lei *et al* J. Bacterol., vol. 169: pp. 4379, 1987). Alternatively, protein may be allowed to form inclusion bodies, and subsequently be ~~resolubilized~~ resolubilized and refolded (Kim S. H. *et al.* ~~Mol. Immunol.~~ Mol. Immunol., vol. 34: pp. 891, 1997).

Please replace the paragraph at page 55, lines 1-11, with the following amended paragraph:

Point mutations can be introduced into the DNA encoding the polypeptide, or one, any, both, several or all of the polypeptides of a complex by any method known in the art, such as ~~oligonucleotide-mediated~~ oligonucleotide-mediated site-directed

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mutagenesis. Such methods may utilize oligonucleotides that are homologous to the flanking sequences of such codons, but that encode tyrosine at the selected site or sites. With these oligonucleotides, DNA fragments containing the point mutation or point mutations are amplified and inserted into the gene or genes, for example, by subcloning. One example of such methods is the application of the QuikChange™ Site-Directed Mutagenesis Kit (~~Stratagene~~, Stratagene, Catalog # 200518); this kit uses the Pfu enzyme having non-strand-displacing action in any double stranded plasmid mutation in PCR reactions. Other methods may utilize other enzymes such as DNA polymerases, or fragments and/or analogs thereof.

Please replace the paragraph at page 56, lines 2-32, with the following amended paragraph:

Partially purified polypeptides containing appropriate tyrosine residues may be equilibrated by dialysis in a buffer, such as phosphate buffered saline (PBS), together or separately before mixing them. The catalyst is then added (on ice or otherwise). The catalyst of the reaction is any compound that will result in the above cross-link reaction. The catalyst should have the structural components that convey the specificity of the reaction, generally provided by a structure complexing a metal ion, and the ability to abstract an electron from the substrate in the presence of an oxidizing reagent, generally provided by the metal ion. An active metal is encased in a stable ligand that blocks non-specific binding to chelating sites on protein surfaces. For example, either a metalloporphyrin, such as, but not limited to, 20-tetrakis (4-sulfonateophenyl)-21H,23H-porphine manganese (III) chloride (MnTPPS) or hemin iron (III) protoporphyrin IX chloride (Campbell L. A. *et al.* Bioorganic and Medicinal Chemistry, vol. 6: pp. 1301-1037, 1998), or a metal ion-peptide complex, such as the tripeptide NH₂-Gly-Gly-His-COOH complexing Ni⁺⁺ can serve as the catalyst of the

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reaction. Metalloporphyrins are a class of oxidative ligand-metal complexes for which there are few, if any, high affinity sites in naturally occurring eukaryotic proteins. The reaction can also be catalyzed by intramolecular Ni⁺⁺ peptide complexes, such as--and C-terminal amino acids consisting either of 3 or more histidine residues (his-tag), or of the above GGH tripeptide. The reaction is initiated by the addition of the oxidizing reagent at room temperature or otherwise. Oxidizing reagents include, but are not limited to, hydrogen peroxide, oxone, and magnesium monoperoxypthalic acid hexahydrate (MMPP) (Brown K. C. *et al.* Biochem.; vol. 34(14): pp. 4733-4739, 1995). Higher specificity can be achieved by using a photogenerated oxidant, such as the oxidant used in the process described by Fancy D. and Thomas Kodadek, which involves brief photolysis of tris-bipyridylruthenium(II) dication with visible light in the presence of an electron acceptor, such as ammonium persulfate (Fancy D. A. and Kodadek T. Proc. Natl. Acad. Sci., U.S.A.; vol. 96: pp. 6020-24, 1999). The optimal reaction period is preferably determined for each application; however, in cases where an optimization process is not possible, the reaction should preferably be stopped after one minute. Using a photogenerated oxidant, such as above described, the exposure to light can be less than one second. The reaction is stopped by the addition of a sufficient amount of reducing agent, such as ~~β-mercaptoethanol~~ β-mercaptoethanol, to counteract and/or ~~neutralize~~ neutralize the oxidizing agent.

Please replace the paragraph at page 57, lines 24-31, with the following amended paragraph:

The polypeptides now containing tyrosyl side-chains at the residues to which the cross-link reaction should be directed are subjected to the cross-link reaction under the conditions determined as described above and carried out, also as described above. The efficiency of the reaction may be examined, for example, by Western blotting

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experiments, in which a cross-linked complex should run at approximately the molecular weight of both or all polypeptides of the complex. ~~If~~, If the bond is readily formed under the above conditions, the strength of the reaction may still be further adjusted to the minimally required strength.